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(54) Title: THERAPEUTIC USE OF MYELIN-ASSOCIATED GLYCOPROTEIN (MAG)

(57) Abstract

The present invention relates to the use of a myelin associated glycoprotein (MAG) for the regulation of growth of neurons in peripheral nervous system (PNS) or central nervous system (CNS). The present invention relates to MAG agonists to inhibit neuron growth and to MAG antagonists to suppress the inhibition of neuron growth. The present invention also relates to an assay method useful to identify MAG antagonist agents that suppress inhibition of neuron growth, comprising the steps of: (a) culturing neurons on a growth permissive substrate that incorporates a growth-inhibiting amount of MAG; and (b) exposing the cultured neurons of step (a) to a candidate MAG antagonist agent in an amount and for a period sufficient prospectively to permit growth of said neurons; thereby identifying as MAG antagonists said candidates of step (b) which elicit neurite outgrowth from said cultured neurons of step (a).

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THERAPEUTIC USE OF MYELIN-ASSOCIATED GLYCOPROTEIN (MAG)

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to the use of a myelin associated glycoprotein (MAG) for the regulation of growth of neurons in peripheral nervous system (PNS) or central nervous system (CNS).

(b) Description of Prior Art

Neurons injured in the central nervous system (CNS) of mammals do not regenerate. A major barrier to axonal regeneration in the CNS of mammals is the presence of growth inhibitory molecules. It is not known whether there is one or several inhibitory proteins present in myelin membranes, and the primary sequence has not yet been identified for any myelin associated protein inhibitor. The axon growth inhibitory property of CNS myelin has been well established, but the inhibitory proteins remain unknown. It is well documented that myelin in the CNS of adult mammals inhibits the growth of neurons *in vitro* (P. Caroni, M.E. Schwab, (1988) *Neuron*, **1**:85-96; M.E. Schwab, P. Caroni, (1988) *J. Neurosci.*, **8**:2382-2393; M.E. Schwab, *Annu. Rev. Neurosci.*, (1993) **16**:565; C. Bandtlow et al., (1990) *J. Neurosci.*, **10**:3838-3848).

Inhibitory activity can be extracted from SDS gels with proteins of approximately 250 and 35 kDa. Further, many *in vivo* experiments suggest that myelin-derived inhibitors block regeneration *in vivo* (Schnell and Schwab, (1990) *Nature*, **243**:268; Schnell et al., (1994) *Nature*, **367**:170).

In addition, molecules secreted by oligodendrocytes, that have growth inhibitory properties, have been identified as 160 and 180 kDa homologues of tenascin (Peshiva et al., (1989) *JCB*, **109**:1765-1788; Fuss et al., (1993) *JCB*, **120**:1237-1249; Lochter et

al., (1991) *JCB*, **113**:1159-1171). The tenascin-like proteins are secreted and, therefore, are not expected to be present in myelin.

Of the present candidates, two proteins are antigenically related, and two others are secreted and do not likely co-purify with myelin (P. Pesheva et al., *J. Cell Biol.* **109**, 1765 (1989); B. Fuss et al., *J. Cell Biol.* **5**, 1237 (1993)).

To date none of the inhibitory proteins in myelin have been identified at the molecular level.

It would be highly desirable to be provided with such a myelin-derived inhibitory protein to be able to find reagents to overcome growth inhibition which would allow regenerative regrowth of damaged axons in the mammalian CNS.

SUMMARY OF THE INVENTION

There is now provided evidence for a novel myelin-derived inhibitory protein which can block regeneration of damaged neurons in the mammalian CNS and PNS.

In accordance with the present invention, another protein has now been identified, myelin associated glycoprotein (MAG) as one of the major molecular components involved in contact-mediated growth inhibition on myelin. The growth inhibitory property of MAG was determined by: 1) demonstrating that MAG chromatographed with partially purified myelin-derived inhibitory activity; 2) testing the ability of recombinant MAG to inhibit neurite growth.

In accordance with the present invention, there is provided the use of a myelin associated glycoprotein (MAG) for the regulation of growth of neurons in PNS or CNS.

In accordance with another aspect of the present invention, there is provided a method to suppress the inhibition of neuron growth, comprising the steps of delivering, to the nerve growth environment, a myelin-associated glycoprotein antagonist in an amount effective to reverse said inhibition.

In accordance with another aspect of the present invention, there is provided an assay method useful to identify MAG antagonist agents that suppress inhibition of neuron growth, comprising the steps of:

a) culturing neurons on a growth permissive substrate that incorporates a growth-inhibiting amount of MAG; and

b) exposing the cultured neurons of step a) to a candidate MAG antagonist agent in an amount and for a period sufficient prospectively to permit growth of the neurons;

thereby identifying as MAG antagonists the candidates of step b) which elicit neurite outgrowth from the cultured neurons of step a).

In a further aspect of the present invention, there is provided a method for inhibiting neuron growth, comprising the steps of introducing into the growth environment of the neurons a growth inhibiting amount of MAG or a MAG agonist.

For the purpose of the present invention the following terms are defined below.

"Agonist" refers to a pharmaceutical agent having myelin associated glycoprotein biological activity of inhibiting the neurite outgrowth of neurons cultured on a permissive substrate or inhibiting the regeneration of damaged neurons. It would be desirable to inhibit neuron growth in cases of epilepsy, neuroblastoma, and neuromas, a disease state in a mammal which includes neurite outgrowth or other

neural growth of an abnormal sort which causes pain at the end of an amputated limb. Agonists which may be used in accordance with the present invention include without limitation a MAG fragment, an analog of MAG or of the MAG fragment, a derivative of either MAG, the MAG fragment or said analog, an anti-idiotypic MAG antibody or a binding fragment thereof, MAG ectodomain, and a pharmaceutical agent.

"Antagonist" refers to a pharmaceutical agent which in accordance with the present invention which inhibits at least one biological activity normally associated with MAG, that is blocking or suppressing the inhibition of neuron growth. Antagonists which may be used in accordance with the present invention include without limitation a MAG antibody or a binding fragment of said antibody, a MAG fragment, a derivative of MAG or of a MAG fragment, an analog of MAG or of a MAG fragment or of said derivative, and a pharmaceutical agent, and is further characterized by the property of suppressing MAG-mediated inhibition of neurite outgrowth.

The agonist or antagonist of MAG in accordance with the present invention is not limited to MAG or its derivatives, but also includes the therapeutic application of all agents, referred herein as pharmaceutical agents, which alter the biological activity of the neuronal receptor for MAG such that growth of neurons or their axons is suppressed. The receptor can be identified with known technologies by those skilled in the art (Mason, (1994) *Curr. Biol.*, **4**:1158-1161) and its association with MAG or fragments thereof can be determined. The neuronal receptor for MAG may or may not be the same as cell surface molecules that recognize and bind MAG in an adhesion assay (Kelm et al., (1994) *Curr. Biol.*, **4**:965-972). Once

the active MAG-recognition domain of the receptor(s) is/are known, appropriate peptides or their analogs can be designed and prepared to serve as agonist or antagonist of the MAG-receptor interaction.

The term "effective amount" or "growth-inhibiting amount" refers to the amount of pharmaceutical agent required to produce a desired agonist or antagonist effect of the MAG biological activity. The precise effective amount will vary with the nature of pharmaceutical agent used and may be determined by one of ordinary skill in the art with only routine experimentation.

As used herein, the terms "MAG biological activity" refers to cellular events triggered by MAG, being of either biochemical or biophysical nature. The following list is provided, without limitation, which discloses some of the known activities associated with MAG: contact-mediated growth inhibition on myelin, inhibition of neuron growth, inhibition of neurite outgrowth, adhesion to neuronal cells, and promotion of neurite outgrowth from newborn dorsal root ganglion neurons.

In general, the abbreviations used herein for designating the amino acids are based on the conventional one-letter abbreviations as indicated below:

Alanine	A
Arginine	R
Asparagine	N
Aspartic Acid	D
Cysteine	C
Glutamine	Q
Glutamic Acid	E
Glycine	G
Histidine	H
Isoleucine	I

Leucine	L
Lysine	K
Methionine	M
Phenylalanine	F
Proline	P
Serine	S
Threonine	T
Tryptophane	W
Tyrosine	Y
Valine	V

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the neurite outgrowth from DiI-labeled NG108-15 cells on myelin and MAG substrates X 160;

Fig. 2 illustrates a quantitative analysis of neurite outgrowth from NG108-15 cells on different substrates; and

Fig. 3 is the analysis of growth inhibition after separation of myelin proteins by DEAE anion exchange chromatography.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, to search for the myelin-associated inhibitory molecules the strategy pursued was the purification of myelin inhibitors by non-denaturing extraction and column chromatography. This approach has allowed the identification of a known protein of unknown function as a major myelin-derived growth inhibitor.

In accordance with the present invention, the myelin-associated glycoprotein (MAG) was identified as a major inhibitory molecules present in myelin. The primary sequence of the protein has previously been reported (Arquint et al., (1987) *PNAS*, **84**:600-604;

Salzer et al., (1987) *JCB*, **104**:957-965). This protein is present in both peripheral nervous system (PNS) and CNS myelin, an observation consistent with findings that PNS myelin also has inhibitory properties (Fig. 2).

Myelin-derived inhibitory activity was assessed by bioassays performed in tissue culture. Cells grow neurites on "permissive" substrates such as a polylysine or laminin substrate (Fig. 1A). Purified bovine CNS myelin used as a tissue culture substrate permitted cell attachment, but inhibited cAMP-induced neurite outgrowth from NG108-15 cells (Fig. 1B). Cells grown on 4 µg of myelin were rounded and did not grow neurites (Fig. 1B). When cells were grown on recombinant MAG they did not spread or differentiate neurites (Fig. 1C; Attia S. et al., (1993) *J. Neurochem.*, **61**:718-726), but the treatment of MAG with heat at 80°C for 1 hour abolished its inhibitory properties (Fig. 1D). Bovine corpus callosum or sciatic nerve was homogenized with a Dounce™ homogenizer, and myelin was purified as described by W.T. Norton et al. ((1973) *J. Neurochem.*, **21**:749-757). Myelin with 4µg or 8µg protein was added to polylysine-coated 96 well plates, dried and washed with PBS before plating the cells. NG108 cells readily accessible to those skilled in the art, a cell line that extends neurites in response to cAMP (D.G. Puro et al., (1976) *Proc. Natl. Acad. Sci.*, **73**:3544-3548), were plated at 1000 cells/well in triplicate. After 24 hours the cultures were fixed with 4% paraformaldehyde, 0.1% glutaraldehyde, stained with 0.5% cresyl violet, and the percentage of cells with neurites of 1 cell body diameter or longer was determined. In some experiments neurite outgrowth was assayed with Di-I labeled NG108 cells as follows.

cAMP primed NG108 cells were labeled with the lipophilic dye Di-I(1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) by incubating the cultures with 1% solution of the dye in culture medium for 2 hr. The cells were then rinsed and maintained in culture for 3 hr. prior to using them for the neurite outgrowth assay. DiI-labeled NG108 cells were added to the wells at a density of 1000 cells/well, and cultured for 24 hr. in Dulbecco's minimal essential medium (DMEM) containing 5% fetal bovine serum, 1mM dibutyryl cAMP. The cultures were then fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer for 30 min., washed and stored in buffer, the percentage of NG108 cells extending neurites longer than 1 cell body diameter was estimated with a Zeiss Axiomat™ inverted microscope equipped with fluorescence optics.

On a polylysine substrate, 70% ± 2. (n=26) of the plated cells extended neurites compared with 2% ± 0.6 (n=17) on 8µg myelin and 4% ± 1 (n=15) on 4µg myelin.

To characterize the growth inhibitory molecules present in CNS myelin, myelin-derived inhibitory activity was solubilized in octylglucoside and chromatographed on a DEAE anion exchange column.

Bovine brain myelin was extracted for 2 hr. at 20°C with 1% octylglucoside (1 ml per mg of protein) in 0.2 M phosphate buffer (pH 6.8) containing 0.1 M Na_2SO_4, 1 mM EDTA, 1 mM dithiothreitol and a cocktail of protease inhibitors. The extract was clarified by centrifugation at 400,000 g.min. and applied to a column of DEAE-Sepharose™ (Pharmacia; 1.3 cm X 10 cm). After several washes, elution was effected with a NaCl gradient (0.2 M to 2.0 M) containing 1% octylglucoside, 50 mM Tris HCl (pH 7) and 1 mM dithiothreitol.

Fractions (2.0 ml) were collected and protein concentrations were estimated by the Bradford protein assay (Bio-Rad. Toronto, Ont). Aliquots of 70 µl were tested in culture. Proteins were prepared for SDS-PAGE by precipitation with trichloracetic acid, removal of detergent with acetone, and dispersion in boiling sample buffer.

Several peaks of inhibitory activity were eluted with a salt gradient. A profile of growth inhibition present in the column fractions is shown in Fig. 3. Percent inhibition was calculated as (C - E/C x 100) where C was the average number of cells with neurites on polylysine substrates, and E was the average number of cells with neurites on the test substrate. Western blots of the inhibitory fractions with anti-MAG antibody (GenS3; Nobile-Orazio et al., (1984) *Neurology*, **34**:1336-1342) showed that the inhibitory peaks that eluted in low salt corresponded to the elution profile for MAG (Fig. 3). Fig. 3 is a Western blots of column fractions probed with anti-MAG antibody. The inhibitory activity eluted at the highest salt concentrations (Fig. 3, fractions 23-26) was not enriched in MAG, and likely represents a different inhibitory protein activity, possibly the one reported by Schwab (M.E. Schwab, (1993) *Annu. Rev. Neurosci.*, **16**:565).

These data suggested that MAG may be an inhibitor of axon growth. Direct evidence for this role was obtained by showing that recombinant MAG was a potent inhibitor of neurite outgrowth from NG108 cells (Fig 1C; Fig. 2). Heating recombinant MAG at 80°C for 1 hr. abolished this activity (Fig. 1D; Fig. 2). Comparison of cells grown on polylysine, CNS myelin, peripheral nerve myelin, recombinant MAG (MAG), denatured MAG (denat. MAG), and bovine serum albumin (BSA)

demonstrate the potent growth inhibition of PNS and CNS, myelin and MAG. Results are the mean of 3 experiments done in triplicate; 2 experiments for denatured MAG (Fig. 2). In these experiments, no cells extended neurites when plated on MAG compared to 73%± 4 on denatured MAG, or to 69%± 4 on BSA, used as a control. Because MAG is a component of myelin produced by both Schwann cells and CNS glial cells, neurite growth inhibition present in peripheral nerve and CNS myelin were compared. Purified bovine CNS and peripheral nerve myelin plated at 4μg protein/well inhibited neurite outgrowth equally (Fig. 2). The observed growth inhibition by MAG, and its presence in peripheral nerve myelin, accords with recent observations that unlesioned sciatic nerve has growth inhibitory properties (K.S. Bedi et al., (1992) *Europ. J. Neurosci*, **4**:193).

Although MAG is reported here as having potent neurite growth-inhibitory activity, its sequence homology with adhesion molecules of the immunoglobulin family has led others to investigate a possible role for MAG in cell adhesion (M. Arquint et al., (1987) *Proc. Natl. Acad. Sci. U.S.A.*, **84**:600; M. Poltorak et al., (1987) *J. Cell Biol.*, **105**:1893; J.L. Salzer et al., (1987) *J. Cell Biol.*, **104**:957; Kelm, S. et al., (1994) *Current Biology*, **4**:965-972; Schachner et al., (1994) *Curr. Opinion Neurobiol.*, **4**:726-734; Doherty et al., (1994) *Curr. Opinion Neurobiol.*, **4**:49-55).

The results of the present invention were correlated as follows with the growth inhibitory properties of MAG with experimental evidence that the MAG extracellular domain may mediate adhesion to axons. First, the finding that recombinant MAG lacking the L2/HNK-1 carbohydrate epitope is a potent neurite outgrowth inhibitor suggests that there may be functional

differences among carbohydrate variants of MAG. Further, observations that MAG-containing liposomes bind to neurons specifically suggests a receptor-mediated interaction between neurons and MAG. However, such experiments do not reveal how MAG binding may affect growth cone dynamics. Finally, adhesive properties of MAG have been examined when it was expressed in heterologous, living cells. In these experiments, cell adhesion molecules or extracellular matrix components that are known to override the growth inhibition on myelin may be present.

The growth inhibitory properties of MAG may be of critical importance after nerve injury in the CNS. In contrast to injured peripheral nerves, myelin debris in the CNS is not removed very quickly after injury (G. Stoll et al., (1989) *J. Neurosci.*, **9**:2327-2335). In addition, peripheral nerve myelin is associated with molecules such as laminin that can override the inhibitory effects of myelin. Although it is known that CNS neurons will regenerate in a peripheral nerve environment, they show little capacity to elongate in the CNS. Growth inhibition by MAG, which is a prominent glycoprotein component of the total myelin protein (R.H. Quarles, D.R. Coleman, J.L. Salzer and B.D. Trapp., (1992) In: *Myelin: Biology and Chemistry*, R.E. Martenson, ed. CRC Press), may thus contribute significantly to the failure of injured axons to regrow within the CNS.

Based on the present evidence that MAG is a major growth inhibitory protein in myelin, one can now identify agents and therapies that suppress MAG-mediated inhibition of nerve growth. Further, one can exploit the growth inhibiting properties of MAG, or MAG agonists, to suppress undesired nerve growth. Without the critical finding that MAG has growth

inhibitory properties, these strategies would not be developed.

More particularly, the invention provides an assay method adapted to identify MAG antagonist, that is agents that block or suppress the growth-inhibiting action of MAG. In its most convenient form, the assay is a tissue culture assay that measures neurite outgrowth as a convenient end-point, and accordingly uses nerve cells that extend neurites when grown on a permissive substrate. Nerve cells suitable in this regard include neuroblastoma cells of the NG108 lineage, such as NG108-15, as well as other neuronal cell lines such as PC12 cells (American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852 USA, ATCC accession No. CRL 1721), human neuroblastoma cells, and primary cultures of CNS or PNS neurons taken from embryonic, postnatal or adult animals. The nerve cells, for instance about 10^3 cells-microwell or equivalent, are cultured on a growth permissive substrate, such as polylysine or laminin, that is overlayed with a growth-inhibiting amount of MAG. The MAG incorporated in the culture is suitably myelin-extracted MAG, although forms of MAG other than endogenous forms can be used provided they exhibit the MAG property of inhibiting neuron growth when added to a substrate that is otherwise growth permissive. Suitable MAG alternatives include, for example, the MAG ectodomain reported by Salzer et al. ((1989) *Develop. Neurosci.*, **11**:377-390) and by Attia et al. ((1993) *J. Neurochem.*, **61**:718-726), and other growth-inhibiting MAG fragments.

In this assay, candidate MAG antagonists, i.e., compounds that block the growth-inhibiting effect of MAG, are added to the MAG-containing tissue culture preferably in amounts sufficient to neutralize the MAG

growth-inhibiting activity, that is between 1.5 and 15 μg of MAG antagonists per well containing a density of 1000 NG108-15 cells/well cultured for 24 hr. in Dulbecco's minimal essential medium. After culturing for a period sufficient for neurite outgrowth, e.g. 3-7 days, the culture is evaluated for neurite outgrowth, and MAG antagonists are thereby revealed as those candidates which elicit neurite outgrowth. Desirably, candidates selected as MAG antagonists are those which elicit neurite outgrowth to a statistically significant extent, e.g., in at least 50%, more desirably at least 60%, e.g. 70%, per 1,000 cultured neurons.

Other assay tests that could be used include without limitation the following: 1) The growth cone collapse assay that is used to assess growth inhibitory activity of collapsin (Raper, J.A., and Kapfhammer, J.P., (1990) *Neuron*, **2**:21-29; Luo et al., (1993) *Cell*, **75**:217-227) and of various other inhibitory molecules (Igarashi, M. et al., (1993) *Science*, **259**:77-79) whereby the test substance is added to the culture medium and a loss of elaborate growth cone morphology is scored. 2) The use of patterned substrates to assess substrate preference (Walter, J. et al., (1987) *Development*, **101**:909-913; Stahl et al., (1990) *Neuron*, **5**:735-743) or avoidance of test substrates (Ethell, D.W. et al., (1993) *Dev. Brain Res.*, **72**:1-8). 3) The expression of recombinant proteins on a heterologous cell surface, and the transfected cells are used in co-culture experiments. The ability of the neurons to extend neurites on the transfected cells is assessed (Mukhopadhyay et al., (1994) *Neuron*, **13**:757-767). 4) The use of sections of tissue, such as sections of CNS white matter, to assess molecules that may modulate growth inhibition (Carbonetto et

al., (1987) *J. Neuroscience*, **7**:610-620; Savlo, T. and Schwab, M.E., (1989) *J. Neurosci.*, **9**:1126-1133). 5) Neurite retraction assays whereby test substrates are applied to differentiated neural cells for their ability to induce or inhibit the retraction of previously extended neurites (Jalnink et al., (1994) *J. Cell Biol.*, **126**:801-810; Suidan, H.S. et al., (1992) *Neuron*, **8**:363-375; Smalheiser, N. (1993) *J. Neurochem.*, 61:340-343). 6) The repulsion of cell-cell interactions by cell aggregation assays (Kelm, S. et al., (1994) *Current Biology*, **4**:965-972; Brady-Kainay, S. et al., (1993) *J. Cell Biol.*, **4**:961-972). 7) The use of nitrocellulose to prepare substrates for growth assays to assess the ability of neural cells to extend neurites on the test substrate (Laganeur, C. and Lemmon, V., (1987) *PNAS*, **84**:7753-7757; Dou, C-L and Levine, J.M., (1994) *J. Neuroscience*, **14**:7616-7628).

Useful MAG antagonists include antibodies to MAG and the binding fragments of those antibodies. Antibodies which are either monoclonal or polyclonal can be produced which recognize MAG and its various epitopes using now routine procedures. For the raising of antibody, various host animals can be immunized by injection with MAG or fragment thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinmitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin).

A monoclonal antibody to an epitope of MAG can be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Köhler and Milstein ((1975) *Nature*, **256**:495-497), and the more recent human B cell hybridoma technique (Kozbor et al., (1983) *Immunology Today*, **4**:72) and EBV-hybridoma technique (Cole et al., (1985) In *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96). In a particular embodiment, the procedure described by Nobile-Orazio et al. ((1984) *Neurology*, **34**:1336-1342) may be used to obtain antibodies which recognize recombinant MAG (Attia S. et al., (1993) *J. Neurochem.*, **61**:718-726).

The monoclonal antibodies for therapeutic use may be human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies. Human monoclonal antibodies may be made by any of numerous techniques known in the art (e.g. Teng et al., (1983) *Proc. Natl. Acad. Sci. U.S.A.*, **80**:7308-7312; Kozbor et al., (1983) *Immunology Today*, **4**:72-79; Olsson et al., (1982) *Meth. Enzymol.*, **92**:3-16). Chimeric antibody molecules may be prepared containing a mouse antigen-binding domain with human contact regions (Morrision et al., (1984) *Proc. Natl. Acad. Sci. U.S.A.*, **81**:6851; Takeda et al., (1985) *Nature*, **314**:452).

A molecular clone of an antibody to a MAG epitope can be prepared by known techniques. Recombinant DNA methodology may be used to construct nucleic acid sequences which encode a monoclonal antibody molecule, or antigen binding region thereof (see e.g., Maniatis et al., (1982) In *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

For use, MAG antibody molecules may be purified by known techniques, such as immunoabsorption or immunoaffinity chromatography, chromatographic methods such as HPLC (high performance liquid chromatography), or a combination thereof, etc.

MAG antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F $(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab, fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, and the two Fab or Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

Monoclonal antibodies known to react with human MAG may be tested for their usefulness to serve as MAG antagonists (Nobile-Orazio et al., (1984) *Neurology*, **34**:1336-1342; Doberson et al., (1985) *Neurochem. Res.*, **10**:499-513).

Also suitable as MAG antagonist candidates for evaluation in the assay are fragments, analogs and derivatives of MAG. Such candidates may interfere with MAG-mediated growth inhibition as competitive but non-functional mimics of endogenous MAG. From the reported amino acid sequence of MAG and from the cloned DNA coding for it, it will be appreciated that MAG fragments can be produced either by peptide synthesis or by recombinant DNA expression of either a truncated MAG, such as the 67kD ectodomain described by Attia S. et al. ((1993) *J. Neurochem.*, **61**:718-726) or of 72kD intact MAG could be prepared using standard recombinant procedures, that can then be digested enzymically in either a random or a site-selective manner. Analogs of MAG or MAG fragments can be gener-

ated also by recombinant DNA techniques or by peptide synthesis, and will incorporate one or more, e.g. 1-5, L- or D-amino acid substitutions. Derivatives of MAG, MAG fragments and MAG analogs can be generated by chemical reaction of the parent substance to incorporate the desired derivatizing group, such as N-terminal, C-terminal and intra-residue modifying groups that have the effect of masking or stabilizing the substance or target amino acids within it.

In specific embodiments of the invention, candidate MAG antagonists include those that are derived from a determination of the functionally active region(s) of MAG contained within, but not exclusively limited to, the known immunoglobulin-like domains. The antibodies mentioned above and any others to be prepared against epitopes in the ectodomain, when found to be function-blocking in *in vitro* assays, can be used to map the active regions of the polypeptide as has been reported (Fahrig et al., (1993) *Europ., J. Neurosci.*, **5**:1118-1126; Tropak et al., (1994) *J. Neurochem.*, **62**:854-862). Thus, it can be determined which of the five immunoglobulin-like domains of MAG is/are recognized by neuronal receptors and/or are involved in inhibition of neurite outgrowth. When those are known, synthetic peptides can be prepared to be assayed as candidate antagonists of the MAG effect. Derivatives of these can be prepared, including those with selected amino acid substitutions to provide desirable properties to enhance their effectiveness as antagonists of the MAG candidate functional regions of MAG can also be determined by the preparation of altered forms of the MAG ectodomain using recombinant DNA technologies to produce deletion or insertion mutants that can be expressed in various cell types as chimaeric proteins that contain the Fc

portion of immunoglobulin G (Kelm et al., (1994) *Curr. Biol.*, **4**:965-972). Alternatively, candidate mutant forms of MAG can be expressed on cell surfaces by transfection of various cultured cell types. All of the above forms of MAG, and forms that may be generated by technologies not limited to the above, can be tested for the presence of functional regions that inhibit or suppress neurite outgrowth, and can be used to design and prepare peptides to serve as antagonists.

In accordance with an aspect of the invention, the MAG antagonist is formulated as a pharmaceutical composition which contains the MAG antagonist in an amount effective to suppress MAG-mediated inhibition of nerve growth, in combination with a suitable pharmaceutical carrier. Such compositions are useful, in accordance with another aspect of the invention, to suppress MAG-inhibited nerve growth in patients diagnosed with a variety of neurological disorders, conditions and ailments of the PNS and the CNS where treatment to increase neurite extension, growth, or regeneration is desired, e.g., in patients with nervous system damage. Patients suffering from traumatic disorders (including but not limited to spinal cord injuries, spinal cord lesions, surgical nerve lesions or other CNS pathway lesions) damage secondary to infarction, infection, exposure to toxic agents, malignancy, paraneoplastic syndromes, or patients with various types of degenerative disorders of the central nervous system (Cutler, (1987) In: *Scientific American Medicines*, vol. 2, Scientific American Inc., N.Y., pp. 11-1-11-13) can be treated with such MAG antagonists. Examples of such disorders include but are not limited to Strokes, Alzheimer's disease, Down's syndrome, Creutzfeldt-Jacob disease, kuru, Gerstman-Straussler

syndrome, scrapie, transmissible mink encephalopathy, Huntington's disease, Riley-Day familial dysautonomia, multiple system atrophy, amyotropic lateral sclerosis or Lou Gehrig's disease, progressive supranuclear palsy, Parkinson's disease and the like. The MAG antagonists may be used to promote the regeneration of CNS pathways, fiber systems and tracts. Administration of antibodies directed to an epitope of MAG, or the binding portion thereof, or cells secreting such antibodies can also be used to inhibit MAG function in patients. In a particular embodiment of the invention, the MAG antagonist is used to promote the regeneration of nerve fibers over long distances following spinal cord damage.

In accordance with another aspect of the invention, MAG and related compounds that retain the MAG property of inhibiting neuron growth (herein referred to as MAG agonists) are used therapeutically to treat conditions in which suppression of undesired neuronal growth is desired. These include for example the treatment of tumors of nerve tissue and of conditions resulting from uncontrolled nerve sprouting such as is associated with epilepsy and in the spinal cord after nerve injury. In one embodiment, patients with neuroblastoma, and particularly with neuropathies associated with circulating MAG antibody, can be treated with MAG or MAG agonist.

Useful for nerve growth suppression are pharmaceutical compositions that contain, in an amount effective to suppress nerve growth, either MAG or a MAG agonist in combination with an acceptable carrier. MAG can be obtained either by extraction from myelin as described above or, more practically, by recombinant DNA expression of MAG-encoding DNA in the manner reported by Attia S. et al. ((1993) *J. Neurochem.*,

61:718-726). Useful MAG agonists are those compounds which, when added to the permissive substrate described above, suppress the growth of neuronal cells. Particularly useful MAG agonists are those compounds which cause a statistically significant reduction in the number of neuronal cells that extend neurites, relative to control cells not exposed to the agonist. Candidate MAG agonists include fragments of MAG that incorporate the ectodomain, including the ectodomain per se and other N- and/or C-terminally truncated fragments of MAG or the ectodomain, as well as analogs thereof in which amino acids, e.g. from 1 to 10 residues, are substituted, particularly conservatively, and derivatives of MAG or MAG fragments in which the N- and/or C-terminal residues are derivatized by chemical stabilizing groups. Such MAG agonists can also include anti-idiotypes of MAG antibodies and their binding fragments.

In specific embodiments of the invention, candidate MAG agonists include specific regions of the MAG ectodomain, and analogs or derivatives of these. These can be identified by using the same technologies described above for identification of MAG regions that serve as inhibitors of neurite outgrowth.

The MAG-related derivatives, analogs, and fragments of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene of protein level. For example, MAG-encoding DNA can be modified by any of numerous strategies known in the art (Maniatis et al., (1982) In *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), such as by cleavage at appropriate sites with restriction endonuclease(s), subjected

to enzymatic modifications if desired, isolated, and ligated *in vitro*.

Additionally, the MAG-encoding gene can be mutated *in vitro* or *in vivo*, for instance in the manner applied for production of the ectodomain, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art can be used, including but not limited to, *in vitro* site-directed mutagenesis (Hutchinson, et al., (1978) *J. Biol. Chem.*, **253**:6551), use of TAB™ linkers (Pharmacia), etc.

For delivery of either MAG, MAG agonist or MAG antagonist, various known delivery systems can be used, such as encapsulation in liposomes or semipermeable membranes, expression by suitably transformed or transfected glial cells, oligodendroglial cells, fibroblasts, etc. according to the procedure knownto those skilled in the art (Lindvall et al., (1994) *Curr. Opinion Neurobiol.*, **4**:752-757). Linkage to ligands such as antibodies can be used to target delivery to myelin and to other therapeutically relevant sites *in vivo*. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, oral, and intranasal routes, and infusion into ventricles or a site of operation (e.g. for spinal cord lesions) or tumor removal. Likewise, cells secreting MAG antagonist activity, for example, and not by way of limitation, hybridoma cells, excapsulated in a suitable biological membrane may be implanted in a patient so as to provide a continuous source of MAG inhibitor.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WE CLAIM:

1. The use of a myelin associated glycoprotein (MAG), biologically active variants or fragments thereof, for the regulation of growth of neurons in PNS or CNS, wherein MAG has an apparent molecular weight of 100 kDa in its glycosylated form, wherein the polypeptide is 67 kDa or 72 kDa, depending on the isoforms, which comprises about 0.1 to 1.0% of total myelin protein.

2. The use of a myelin associated glycoprotein (MAG), biologically active variants or fragments thereof, for raising antibodies or ligands thereof which overcome growth inhibition, wherein MAG has an apparent molecular weight of 100 kDa in its glycosylated form, wherein the polypeptide is 67 kDa or 72 kDa, depending on the isoforms.

3. A method effective to suppress the inhibition of neuron growth, comprising the step of delivering a MAG antagonist to the nerve growth environment in an amount effective to reverse said inhibition.

4. A method according to claim 3, wherein said MAG antagonist is selected from a MAG antibody or a binding fragment of said antibody, a MAG fragment, a derivative of MAG or of a MAG fragment, an analog of MAG or of a MAG fragment or of said derivative, and a pharmaceutical agent, and is further characterized by the property of suppressing MAG-mediated inhibition of neurite outgrowth.

5. A method according to claim 4, wherein said MAG antagonist is a MAG antibody or a binding fragment thereof.

6. A method according to claim 3, 4 or 5, wherein said MAG antagonist is delivered to the growth environment of a CNS neuron requiring growth or regeneration as a result of spinal cord injury, spinal cord lesions, surgical nerve lesions, damage secondary to infarction, infection, exposure to toxic agents and malignancy.

7. A method according to claim 3, 4 or 5, wherein said MAG antagonist is delivered to a patient having a medical condition selected from Strokes, Alzheimer's disease, Down's syndrome, Creutzfeldt-Jacob disease, kuru, Gerstman-Straussler syndrome, scrapie, transmissible mink encephalopathy, Huntington's disease, Riley-Day familial dysautonomia, multiple system atrophy, amyotropic lateral sclerosis or Lou Gehrig's disease, progressive supranuclear palsy, Parkinson's disease.

8. An assay method useful to identify MAG antagonist agents that suppress inhibition of neuron growth, comprising the steps of:

a) culturing neurons on a growth permissive substrate that incorporates a growth-inhibiting amount of MAG; and

b) exposing the cultured neurons of step a) to a candidate MAG antagonist agent in an amount and for a period sufficient prospectively to permit growth of said neurons;

thereby identifying as MAG antagonists said candidates of step b) which elicit neurite outgrowth from said cultured neurons of step a).

9. A method for inhibiting neuron growth, comprising the step of introducing into the neuron growth environment a growth-inhibiting amount of a neuron growth inhibitor selected from MAG and a MAG agonist.

10. A method according to claim 9, wherein said inhibitor is MAG.

11. A method according to claim 10, wherein said inhibitor is a MAG agonist having MAG-biological activity of inhibiting neurite outgrowth from neurons cultured on a permissive substrate, and is selected from a MAG fragment, an analog of MAG or of the MAG fragment, a derivative of either MAG, the MAG fragment or said analog, an anti-idiotypic MAG antibody or a binding fragment thereof, and a pharmaceutical agent.

12. A method according to claim 10, wherein said MAG agonist is the MAG ectodomain.

13. A method according to claim 9, 10, 11 or 12, wherein said inhibitor is delivered to a patient afflicted with a medical condition selected from epilepsy, neuroblastoma and neuromas.

FIG. 1A

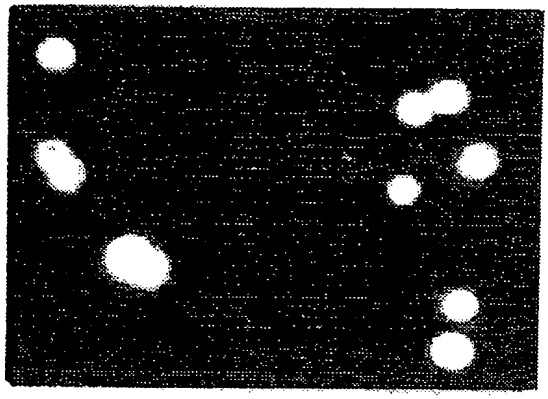


FIG. 1B

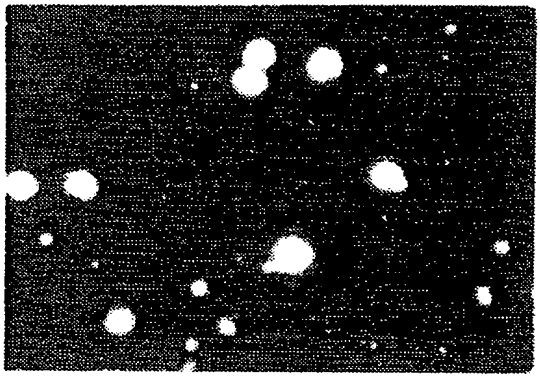


FIG. 1C

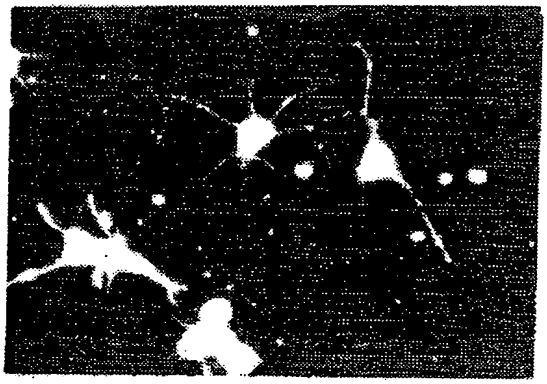


FIG. 1D

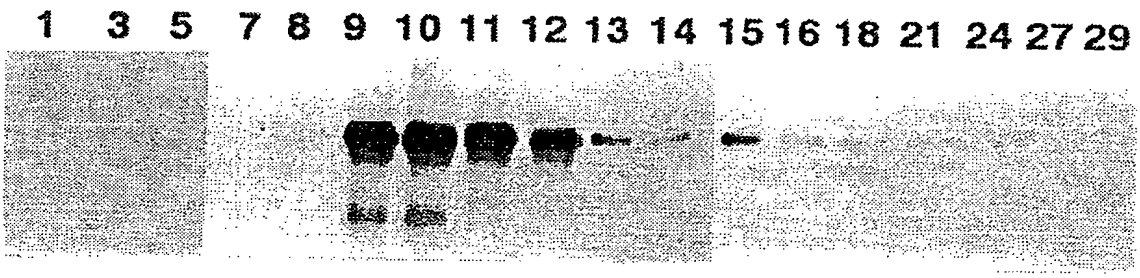


FIG. 3A

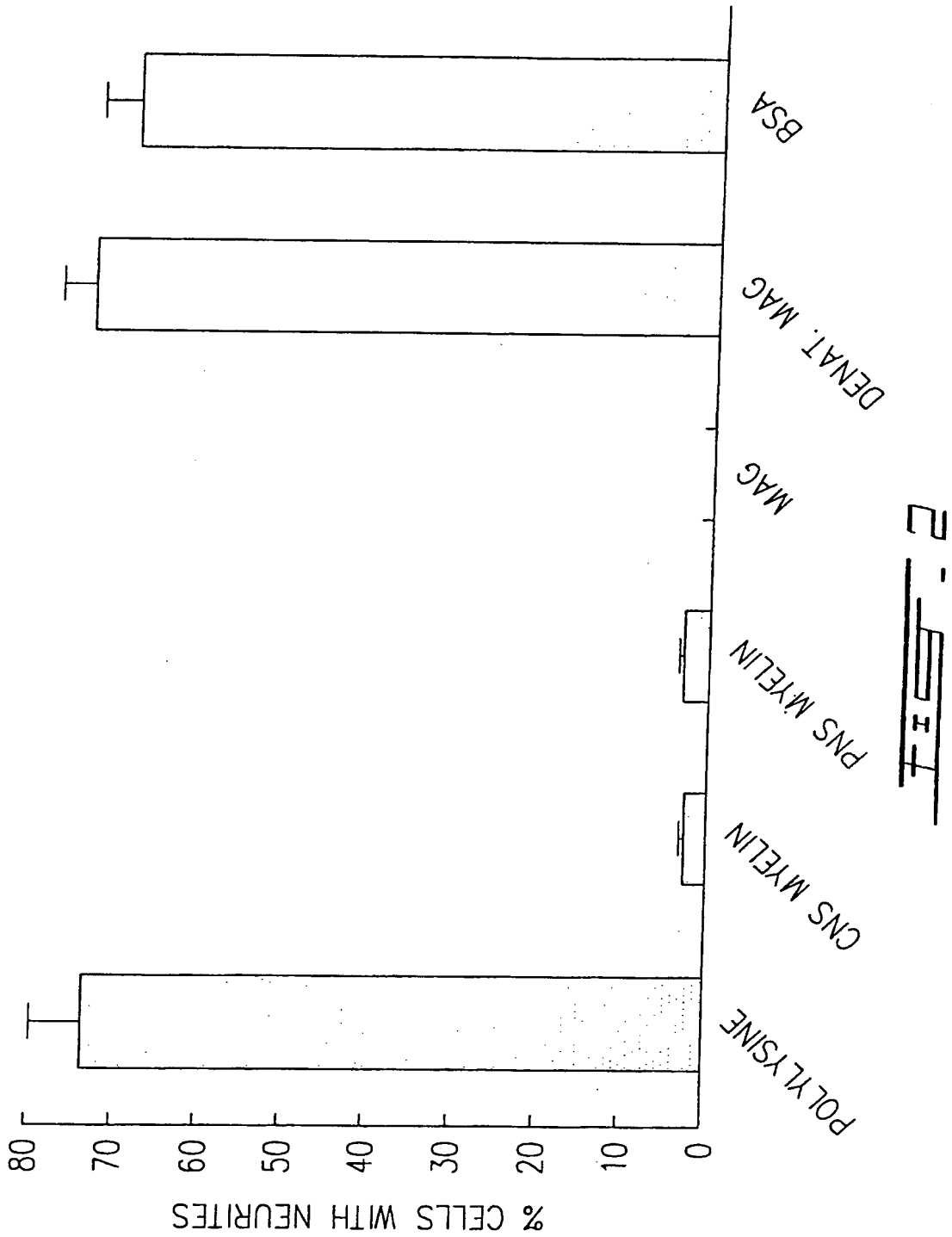
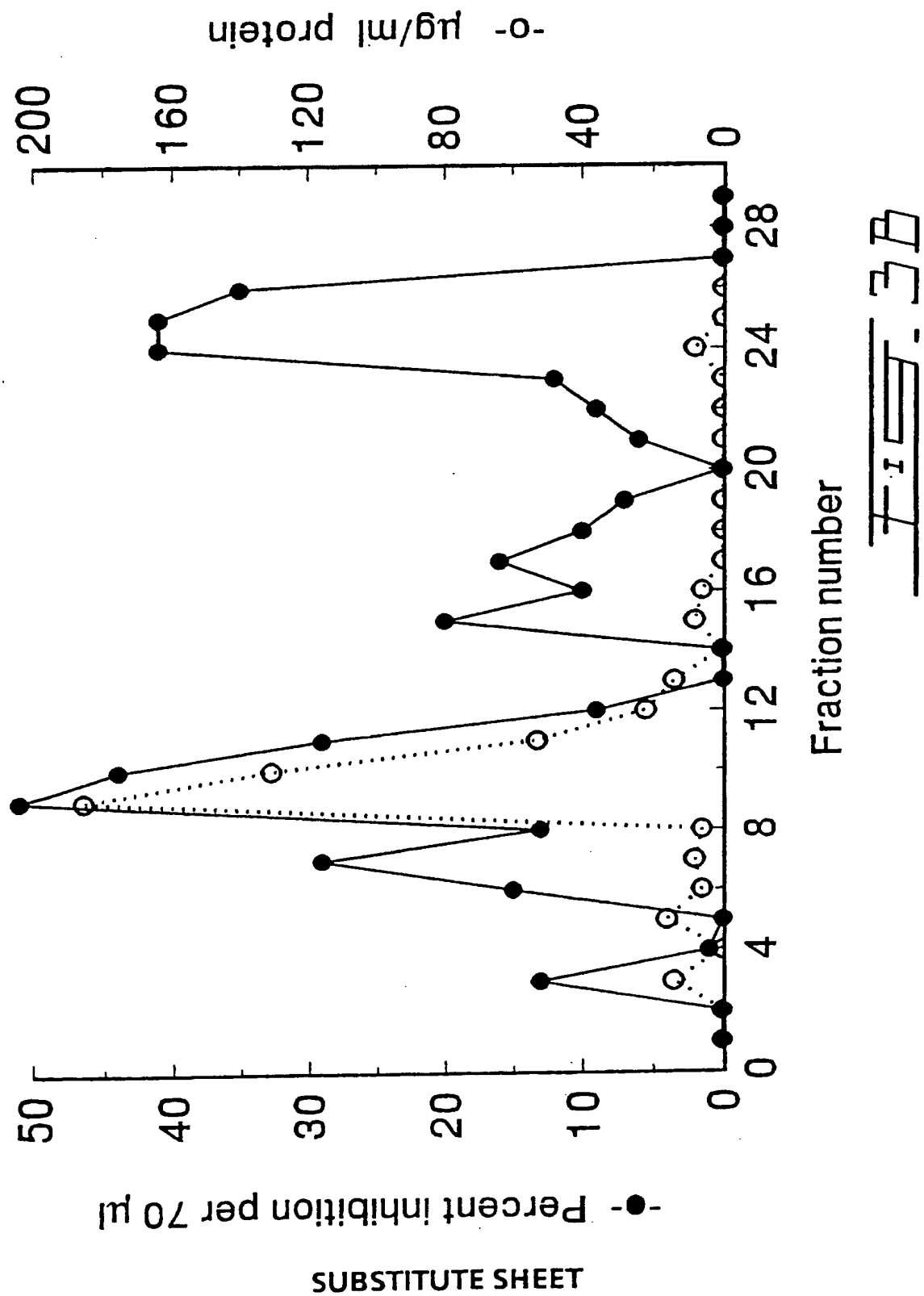

BSA
DENAT. MAG
MAG
PNS MYELIN
CNS MYELIN
POLYLYSINE
0
10
20
30
40
50
60
70
80
% CELLS WITH NEURITES

FIG. 2


-●- Percent inhibition per 70 µl
-o- µg/ml protein
0
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20
30
40
50
0
40
80
120
160
200
0
4
8
12
16
20
24
28
Fraction number

Fig. 3B

INTERNATIONAL SEARCH REPORT

Int ional Application No PCT/CA 95/00089

A. CLASSIFICATION OF SUBJECT MATTER
A 61 K 38/14,A 61 K 39/395,C 12 Q 1/02

According to International Patent Classification (IPC) or to both national classification and IPC 6

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A 61 K,C 12 Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DERWENT ACCESSION no. 93-074 213, Questel Telesystems (WPIL), DERWENT PUBLICATIONS LTD., London; & SU,A,1 721 092 (CYTOLOGY INST.). --	1-7, 11,12
A	NATURE, vol. 311, September 13, 1984, J. KRUSE et al. "Neural cell adhesion molecules and myelin-associated glycoprotein share a common carbohydrate moiety recognized by monoclonal antibodies L2 and HNK-1", pages 153-155, the whole document. ----	1-13

☐ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 May 1995	30.05.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	BÖHM e.h.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Ir ational application No.
PCT/CA 95/00089

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-7,9-13
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 1-7,9-13 are directed to a method of treatment of the human or animal body (Rule 39.1(iv)PCT) the search has been carried out and based on the alleged efforts of the compounds.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.